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Original article

Evaluation of the toxicity and molluscicidal and larvicidal activities of *Schinopsis brasiliensis* stem bark extract and its fractions

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ABSTRACT

Dengue fever and schistosomiasis are major public health issues for which vector control using larvicide and molluscicide substances present in plants provides a promising strategy. This study evaluated the potential toxicity of the extract of hydroethanol *Schinopsis brasiliensis* Engl., Anacardiaceae, stem bark and its chloroform, hexane, ethyl acetate, and hydromethanol fractions against *Artemia salina* and *Aedes Aegypti* larvae and snails *Biomphalaria glabrata*. All of the assays were performed in triplicate and the mean mortality rates were used to determine the LC_{50} and LC_{90} values using the probit method. The hydroethanol hydromethanol extract and fraction were free of toxicity towards *A. salina* (LC_{50} > 1000 µg/ml), while chloroform fraction was moderately toxic (LC_{50} 313 µg/ml); ethyl acetate and hexane fractions displayed low toxicity, with LC_{50} 557 and 582 µg/ml, respectively. Chloroform, hexane, and ethyl acetate fractions showed larvicidal potential towards *A. aegypti* (LC_{50} values of 345, 527 and 583 µg/ml, respectively), while chloroform and ethyl acetate fractions were highly toxic to *B. glabrata* (LC_{90} values of 68 and 73 µg/ml, respectively). Based on these findings, ethyl acetate, chloroform, and hexane fractions should be further investigated for their potential use against the vectors of dengue and schistosomiasis.

Introduction

Aedes aegypti L. is the vector responsible for transmitting the tropical viral infection known as dengue, dengue fever, or breakbone fever. It is considered a major public health concern because it is largely dispersed in urban areas (Porto et al., 2008). Given that 50 to 80 million people are infected with dengue anually in over 100 countries, vector control is extremely important and consists of eliminating breeding sites and applying insecticides (Lingon, 2005; Mendonça et

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al., 2009). However, synthetic insecticides have been observed to have low efficiency due to resistance of insect populations (Barreto, 2005).

Schistosomiasis is another major problem caused by transmission vectors. According to the World Health Organization (WHO), this ancient disease affects indiviuals over 240 million in over 78 countries (WHO, 2013). *Schistosoma mansoni* is the flatworm parasite responsible for most cases of infection. In the countries of South America and Central America, primarily *S. mansoni* uses *Biomphalaria glabrata* snails of the host to an intermediate to complete its life cycle (Rey, 2010). Among the methods used to reduce occurrences of the disease, one promising strategy is the control of populations because malacological eliminating the vector with substances endowed with molluscicide properties interrupts the life cycle of the parasite, and thereby, disease transmission (Santos et al., 2010).

Dengue fever and schistosomiasis are two major public health problems that many countries, including Brazil, are seeking to solve. In this context, the use of plants to control or eradicate these and other diseases is premised on their availability and lower environmental impact, which are variables for obtaining an effective natural product. Plants are important sources of biologically active products that can be responsible for various biological actions, including the larvicidal and molluscicidal properties shown in several studies (Prophiro et al., 2008; Santos et al., 2012).

A previous survey of different parts of plants from northeastern Brazil revealed a high toxicity towards *A. aegypti* and *B. glabrata* (Luna et al., 2005; Oliveira et al., 2006). Although the chemical constituents responsible for causing this toxicity have not been identified, the pharmacological effects of these plants, *e.g.*, molluscicidal and larvicidal activities, are known to be related to the presence of secondary metabolites such as tannins, saponins, terpenoids, steroids, and flavonoids (Treyvaud et al., 2000; Alcanfor et al., 2001; Hymete et al., 2005; Cantanhede et al., 2010).

Schinopsis brasiliensis Engl., Anacardiaceae, popularly known as "baraúna," "braúna," or "quebracho," is used in folk medicine to treat various diseases (Almeida et al., 2010). Biological studies using a methanol leaf extract from *S. brasiliensis* demonstrated its antioxidant, antimicrobial, and anti-inflammatory activities (Ferreira-Junior et al., 2011; Saraiva et al., 2011), while the extract from the plant seed hydroethanol has been shown to have toxic effects against *A. aegypti* larvae (Souza et al., 2011). Therefore, the goal of this study was to evaluate a stem bark extract from *S. brasiliensis* and its fractions for toxicity and molluscicidal and larvicidal activities.

Materials and methods

Collection and identification of plant material

Schinopsis brasiliensis Engl., Anacardiaceae, stem bark was collected in August 2011 in the city of Piranhas in Alagoas, Brazil, whose geographical coordinates were identified by a Garmin Forerunner GPS function (9°35'54.37" S and 37°46'08.31" W). The species was identified by the biologist Marta Maria Cristina Farias and a voucher specimen was deposited in the herbarium of the Department of Biology of the Federal University of Sergipe under the registration voucher number 24442 ASE.

Preparation of the hydroethanol extract and its fractions

The steam bark of *S. brasiliensis* was dried at room temperature, reduced to powder using a slicer, and then extracted by cold maceration with 90% ethanol for five days. The equipament was then filtered and concentrated on a rotatory evaporator (LS Logen Scientific, Lagos, Nigeria) under reduced pressure at 45°C to produce the hydroethanol extract (HEE). The extract (40 g) was dissolved in methanol: water (40% v/v) and successively subjected to liquid-liquid extraction with hexane, chloroform, ethyl acetate and hexane to obtain infor (HxF), chloroform (ChlF), ethyl acetate (EAF), and hydromethanol (HMF) fractions.

Phytochemical screening

Extracts and fractions (5 ml, 1 mg/ml) were qualitatively analyzed by precipitation and colorimetric methods described by Matos (2009) to detect anthocyanins, anthocyanidins, aurones, chalcones, flavanols, flavones, flavonols and xanthones [pH-related color variation by adding sodium hydroxide (3 mol/l) and hydrochloric acid (1 mol/l)], leucoanthocyanidins and catechins (acid-base reactions followed by heating), tannins [ferric chloride precipitation (1 mol/l)], steroids and triterpenoids (Lieberman-Buchard reaction), saponins (Lieberman-Buchard reaction and foam formation) and alkaloids (Dragendorff reaction).

Toxicity assay

The toxic activity of the extract and fractions of *S. brasiliensis* was assessed by the lethality test against *Artemia salina* Leach as described by Meyer et al. (1982) with some modifications. *A. salina* eggs were incubated in cotton-filtered seawater at room temperature for 24 h, after which the larvae were collected with the aid of a light source to attract them. Approximately ten nauplii were transferred to ELISA plate wells containing 1 ml of HEE and the fraction concetrations of solutions at 1, 10, 100, and 1000 µg/ml prepared in seawater containing 1% DMSO. The control group consisted of only the solvent and larvae, and all assays were performed in triplicate. The dead larvae were counted after exposure for 24 h.

Larvicidal assay

Third stage larvae were *A. aegypti* mosquitoes used to evaluate the larvicidal activity of the HEE and its fractions using the methodology described by the World Health Organization (WHO, 2005) modified by the Ribeiro et al. (2009). Larvae were exposed to initially standard solutions of the HEE and its fractions (1000 µg/ml, in distilled water containing 1% DMSO). That ismortalities exceeded 90%, with further tests concentrations lower (100, 250, and 500 µg/ml) were performed to estimate the lethal concentration.

Positive controls were treated with temephos [(O,O'-(thiodi-4,1-phenylene) bis (O,O-dimethylphosphorothiolate)], which was obtained as a commercial sample (3 µg/ml) from Funasa, Brazil. The bioassays were performed in triplicate for each experimental group consisting of 25 larvae, which were placed in contact with 50 ml of each test solution. The mortality or paralysis of the larvae was recorded after 24 and 48 h of exposure. Control tests with distilled water containing 1% DMSO were also performed.

Molluscicide assay

The procedure described by Santos and Sant'Ana (2001) was utilized to evaluate the molluscicidal activity. Test solutions of the HEE and fractions were prepared at different concentrations (25, 50, and 100 µg/ml) using dechlorinated water containing 0.1% DMSO. Treatment with 3 µg/ml of niclosamide® (Oliveira and Paumgartten, 2000) acted to the positive control, while the negative controls were exposed to dechlorinated water containing 0.1% DMSO only. Five snails of *B. glabrata* of uniform size (13-15 mm) were transferred to containers with 250 ml of the test solutions at the established concentrations. The bioassays were performed in triplicate; 24 h after the snails were washed and kept in jars with dechlorinated water and lettuce for feed. The snails examined for lethal effects at 24, 48, 72, and 96 h after exposure, using the absence of motion, muscle contraction, and the change in the shell coloration criteria.

Statistical analysis

From the mortality data obtained in the bioassays, were conducted analyses to estimate the lethal concentrations (LC_{50} and LC_{90}) at a confidence interval of 95%, using the probit analysis method in the Minitab® version 15 statistical software package. Analysis of variance (ANOVA) and Tukey's HSD test were used to determine statistically significant differences between means ($p < 0.05$).

Results and discussions

The qualitative phytochemical screening revealed the presence of flavonols and tannins to the main phenolic compounds in the HEE. When the HEE was partitioned in a gradient of increasing polarity, flavanones, flavanonols, flavonols, tannins, and xanthones were found in the HMF, while the EAF contained aurones, catechins, chalcones, flavanones, saponins, and tannins. The ChlF and HxF showed only triterpenes and steroids, respectively. These findings are in accordance with other studies on the chemical composition of *S. brasiliensis*. In a study by Almeida et al. (2005), the phytochemical analysis of the HEE *S. brasiliensis* from stem bark indicated the presence of phenolic compounds such as quinones, tannins, and triterpenes, and Saraiva et al. (2011) reported the presence of flavonoids and tannins in the hydromethanol extract of the same species leaf. Martín et al. (2010) isolated condensed tannins from the stem bark of a plant of the same genus, *S. balansae*.

According to Bussmann et al. (2011), toxicity levels can be defined the high toxicity ($LC_{50} < 249$ µg/ml), moderate toxicity ($250 < LC_{50} < 499$ µg/ml), low toxicity ($500 < LC_{50} < 1000$ µg/ml), and free of toxicity (1000 µg/ml). Based on this, the results from this study showed that the HEE and HMF were free of toxicity, while ChlF was moderately toxic, and EAF and HxF both had low toxicity (Fig. 1). In a previous study, the HEE from *S. brasiliensis* displayed moderate toxicity towards *A. salina* (LC_{50} 428 µg/ml). However, neither the part of the plant nor the ethanol concentration used for prepararing the extract was provided (Silva et al., 2012). In this study, the activities of the organic fractions obtained from *S. brasiliensis* HEE was incredibly examined

According to Stefanello et al. (2006), natural products from that not exhibit toxicity towards *A. salina* can be tolerated across biological systems. In addition, it has been shown that also toxicity against *A. salina* correlates with insecticide activity for substances with an $LC_{50} < 1000$ µg/ml (Mclaughlin et al., 1995), which corroborates the results in this study in which the fractions with higher larvicidal activity had greater toxicity. Conversely, for plant extracts to be used in natural environments, the low toxicity is considered an interesting feature. Thus, the *A. salina* assay could assist in selecting and monitoring studies of phytochemicals in plant extracts in the search for bioactive substances (Nunes et al., 2008).

When examining the larvicidal activity, only ChlF, HxF, and EAF showed toxicity towards *A. aegypti*, with the LC_{50} value for ChlF being only 60-65% that of the other two fractions (Table 1). HEE and HMF were considered to have no toxicity because they were less than 90% lethal when tested at a concentration of 1000 µg/ml. Cheng et al. (2003) suggested that substances with LC_{50} values less than 100 µg/ml could be considered good larvicidal agents. Even though the fractions did not have LC50 values this low, they showed evident toxicity towards *A. aegypti*, which suggests the presence of chemical constituents with potential larvicidal activity. Further studies to investigate such compounds are needed.

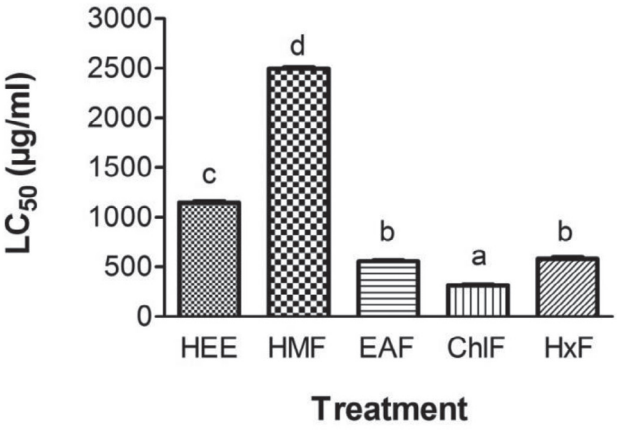

Figure 1 – LC_{50} values for the toxicities of *S. brasiliensis* hydroethanol extract (HEE) and its hexane (HxF), chloroform (ChlF), ethyl acetate (EAF) and hydromethanol (HMF) fractions against *A. salina*. Bars bearing the same lower case letters are not significantly different (ANOVA followed by the Tukey's HSD test at $p < 0.05$).

Table 1
Larvicidal activity of *Schinopsis brasiliensis* fractions towards *A. aegypti* larvae.

Samples	Concentration (µg/ml)	Dead larvae after 48 h[a]	LC (µg/ml)	Confidence Interval 95%
Control[b]	-	0	-	-
Temephos[c]	0.012	75	-	-
ChlF	100	5	LC_{50} 345	341 - 349
	250	28	LC_{90} 895	877 – 916
	500	45		
	1000	73		
HxF	100	3	LC_{50} 527	519 - 534
	250	10	LC_{90} 1.315	1.285 - 1.348
	500	25		
	1000	68		
EAF	100	2	LC_{50} 583	576 - 590
	250	8	LC_{90} 1.333	1.303 - 1367
	500	17		
	1000	68		

[a]All groups consisted of 75 larvae.
[b]Distilled water containing 1% DMSO.
[c]Ribeiro et al., (2009).

Table 2
Molluscicidal activity of *Schinopsis brasiliensis* fractions towards *B. glabrata* snails.

Samples	Concentration (µg/ml)	Dead larvae after 48 h[a]	LC (µg/ml)	Confidence Interval 95%
Control[b]	-	0	-	-
Niclosamide[c]	3	15	LC_{90} 0.10	-
ChlF	25	6	LC_{50} 31	29 - 33
	50	11	LC_{90} 68	62 - 76
	100	15		
EAF	25	3	LC_{50} 39	37 - 42
	50	9	LC_{90} 73	68 - 80
	100	15		

[a]All groups consisted of 15 snails,
[b]Dechlorinated water containing 0.1% DMSO;
[c]LC_{90} value from Oliveira and Paumgartten (2000).

Souza et al. (2012) previously investigated the toxicity of an ethanol extract from *S. brasiliensis* seeds against two strains of *A. aegypti*, with similar results to those obtained in this study. This suggests that the active agents may be distributed in different structures of the plant and in varied concentrations. Among these active agents, triterpenoids, which were detected in ChlF and HxF, have previously been found to have larvicidal activity. Limonoids, which are triterpenoid compounds recognized the insecticides, are growth inhibitors that reduce the reproductive capacity and suppress the appetite of insects (Viegas Junior, 2006). Other studies have found that also tannins and saponins, which are present in EAF, have toxic effects on *A. aegypti* larvae (Silva et al., 2004; Santiago et al., 2005) by adhering to proteins in the midgut epithelial membranes of their cells, causing starvation and death.

In the biological assay to verify the molluscicidal activity, HEE, HMF, and HxF were active only at 100 µg/ml, with less than 90% of the mortality rate, while ChlF and EAF and resulted in 90% mortality at all of the tested concentrations. Although the LC_{90} values for the activities of the ChlF and EAF against *B. glabrata* (Table 2) are not at the same level as Niclosamide®, the results were under 100 µg/ml, which is the maximum LC_{90} value for a substance, extract, or fraction to be considered the potential molluscicidal agent (Adenusi and Odaibo, 2008). In addition, the results for activity against *B. glabrata* and *A. aegypti* suggest that ChlF and EAF and have a higher potential as a molluscicide than as larvicide, because the LC_{90} values were significantly lower against the mollusk.

According to McCullough et al. (1980) and Pieri and Jurberg (1981), molluscicidal agents act by disrupting the osmotic balance of the mollusk, which may cause two responses: one, the release of hemolymph and retraction of the mass cephalopodal into the shell, and two, the abnormal cephalopodal projection of the mass out of the shell. In this study, the first response mechanism was observed during the incubation period. This is the first known study to examine the activity of Schinopsis genera against *B. glabrata*.

Conclusion

The results of this study demonstrated that the ethyl acetate, hexane, and chloroform fractions obtained from the stem bark of *S. brasiliensis* have potential larvicide, while the chloroform and ethyl acetate fractions are toxic to *B. glabrata* snails. Both results can be related to the toxicity of these fractions as demonstrated for *A. salina*. Further investigations of EAF, ChlF, and HxF should be undertaken to isolate the active substances, which may be essential for obtaining more selective and biodegradable compounds to biologically control the vectors for dengue and schistosomiasis.

Authors' contributions

CCSS, DMS and ASD contributed in collecting plant material and preparing herbarium samples. CCSS contributed by performing the biological assays with the help of MISS, ECVA, AYKVS, NPD and ACBL. KALRJ and CKBP contributed by supervising the experiments. SSA, ALLMS and CCSS analyzed the data. CCSS drafted the paper, while BSA, AEGS and CSE contributed to critical reading of the manuscript. All authors read and approved the final manuscript submission.

Conflicts of interest

The authors declare no conflicts of interest.

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